Synergistic effects of histone deacetylase inhibitor in combination with mTOR inhibitor in the treatment of prostate carcinoma

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Abstract. The aim of this study was to elucidate whether the treatment of a prostate carcinoma cell line (LNCaP) and LNCaP-derived tumors with the histone deacetylase (HDAC) inhibitor valproate in combination with the mammalian target of rapamycin (mTOR) inhibitor temsirolimus resulted in synergistic effects on cell proliferation and tumor growth. LNCaP cells were treated with valproate, temsirolimus or a combination of both. The proliferation rates and the expression of key markers of tumorigenesis were evaluated. In *in vivo* experiments, LNCaP cells were implanted into immune-suppressed male nude mice. Mice were treated with valproate (per os), temsirolimus (intravenously) or with a combination of both. Tumor volumes were calculated and mRNA expression was quantified. The incubation of LNCaP cells with the combination of valproate and temsirolimus resulted in a decrease of cell proliferation with an additive effect of both drugs in comparison to the single treatment. In particular, the combined application of valproate and temsirolimus led to a significant upregulation of insulin-like growth factor-binding protein-3 (IGFBP-3), which mediates apoptosis and inhibits tumor cell proliferation. In the mouse model, we found no significant differences in tumor growth between the different treatment arms but immunohistological analyses showed that tumors treated with a combination of valproate and temsirolimus, but not with the single drugs alone, exhibited a significant lower proliferation capacity.

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Abbreviations: LNCaP, lymph node metastasis of prostate cancer; mTOR, mammalian target of rapamycin; IGFBP-3, insulin-like growth factor-binding protein-3; IGF1-R, insulin-like growth factor 1 receptor; ARP, acidic ribosomal protein; PSA, prostate-specific antigen; HDAC, histone-deacetylase; PTEN, phosphatase and tensin homolog; BrdU, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction

Key words: castration-resistant prostate cancer, cell survival signaling, anti-androgen therapy, preclinical study

Introduction

Prostate carcinoma is the most common type of cancer in men in Western countries. At the time of diagnosis, two thirds of carcinoma cases are limited to the prostate and can be treated successfully (1). However, the last third cannot be cured by surgery due to dissemination of the tumor.

The therapy of choice for these patients is a complete deprivation of androgens, resulting in a remission which is, however, limited to 2-4 years, followed by the development of a castration-resistant prostate carcinoma, finally leading to mortality (2).

A key role in promoting the castration-resistant prostate carcinoma is the androgen receptor. This transcription factor in its activated state forms a stable complex with several cofactors and facilitates transcription of distinct genes, which have been shown to be crucial for cell growth and survival (3,4). Androgen deprivation abolishes this activation, leading to cell death or cell cycle arrest. However, treatment with anti-androgens does not result in a permanent regression and cure of the disease. After 2-4 years, increased serum levels of prostate specific antigen (PSA) indicate that the androgen receptor becomes transcriptionally active again (4).

Different pathways are known to reactivate the androgen receptor complex. First, intensified expression of the androgen receptor results in markedly increased protein levels, compensating for the decrease of androgen ligands upon castration. A different possibility is a broadening of the receptor's ligand spectrum by mutations in its coding gene. Finally, altered phosphorylation pathways for the androgen receptor can enhance the receptor activity and even the synthesis of tumor-produced ligands has been described (4).

Therefore, new therapeutic approaches are needed to interfere with carcinoma-specific signal cascades in order to cure this disease.

Various studies investigated the role of histone-deacetylases (HDACs) in prostate carcinoma, which have been shown to be upregulated in these cells, mediating a decreased expression of tumor suppressors (5-8). Blocking HDACs with specific inhibitors leads to an increase in the protein levels of crucial tumor suppressors.

The drug valproate, which has been used for decades in the treatment of epilepsy, has also been shown to have inhibitory effects on HDACs (9).

The treatment of prostate carcinoma cell lines with valproate resulted in an increase in histone acetylation. Furthermore, expression of the tumor suppressor p21 was elevated and the proliferation rate of the treated cells was significantly reduced (10). In previous studies, we showed that the treatment of prostate carcinoma cells (LNCaP) with valproate leads to the induction of apoptosis and an altered gene expression in genes essential for proliferation or apoptosis (11). Functional analyses showed that the increased expression of the estrogen receptor β (ERβ) is responsible for these effects (12).

A further pathway which is involved in cell growth, metabolism and survival is the mammalian target of rapamycin (mTOR) signal cascade. The activity of the mTOR pathway is regulated by various positive and negative signals. Enhancement of mTOR activity is, for instance, mediated by growth factors such as insulin-like growth factor 1 (IGF1) and its receptor (IGF1-R), which activates phosphoinositide-3-kinase (PI3K). PI3K, in turn, is inhibited by the tumor suppressor phosphatase and tensin homolog (PTEN), an essential mediator of apoptosis (13). Mutations in PTEN have been found in various cancer specimens and facilitate proliferation and tumor growth (14-16). PTEN is downregulated in advanced stages of prostate carcinoma (17).

The amount of stimulating IGF1 is controlled by IGF-binding protein-3 (IGFBP-3). IGFBP-3 binds IGF1 and thereby prevents its association with its receptor, resulting in decreased cell proliferation (18). Moreover, IGFBP-3 also exhibits IGF-independent antiproliferative and pro-apoptotic capacities (19,20). Therefore, drugs that increase the expression of IGFBP-3 may potentially inhibit tumor cell proliferation and growth. In this study, we investigated whether a combination of the HDAC-inhibitor valproate and the mTOR inhibitor temsirolimus show synergistic effects on cell proliferation and tumor growth *in vitro* and *in vivo*.

Materials and methods

Cell culture treatment of human tumor cells. LNCaP cells were maintained in RPMI medium, supplemented with 10% fetal calf serum, 2% amino acid solution and 1% L-glutamine. For HDAC-inhibitor stimulation, 100,000 cells were seeded in 6-well petri dishes. After 24 h, cells were treated with 1 mmol/l sodium valproate (Sigma, Taufkirchen, Germany). After 48 h, an additional 1 mmol/l sodium valproate was added. Temsirolimus (Pfizer) treatment was carried out 72 h after cell seeding with a final concentration of 1 mmol/l. Combined treatment was carried out similarly. Five days after seeding, RNA was extracted as described below.

Cell vitality was estimated by the Alamar Blue assay (Biosource, Solingen, Germany), and cell proliferation was analyzed by BrdU-ELISA (Roche Diagnostics GmbH, Mannheim, Germany).

Animal experiments. *In vivo* experiments were approved by the local animal protection committee (Reference 33.9-42502-04-10/0111).

Eight-week-old male athymic nude NMRI$^{nu/nu}$ mice were purchased from Janvier Laboratory, Le Genest Saint Isle, France. For acclimatization, mice were kept for 2 weeks in standard cages with air filter hoods. All animals received a subcutaneous inoculation dorsal of the forelegs of 10^6 LNCaP cells resuspended in 100 µl PBS mixed with 100 µl Matrigel (BD Biosciences, Heidelberg, Germany) through a 26-gauge needle. Subcutaneous tumors were measured twice a week with calipers. Tumor volumes were calculated by the formula: large diameter x (smaller diameter)^2 x0.5 (21). Treatment of the animals was initiated as soon as the tumor volume had reached 120 mm^3. In four different groups, mice were left untreated (control group), or were treated with 800 mg/kg/day valproate (via drinking water), with 0.3 mg/kg temsirolimus intravenously once a week, or with a combination of both drugs by their individual application. Tumor volume was calculated over the period of 7 weeks. Subsequently, animals were sacrificed and tumor samples were excised and stored in RNAlater (Qiagen, Hilden, Germany) for mRNA measurements or in 4% formaldehyde for histology.

mRNA-expression analysis. Total cellular RNA from LNCaP cells was extracted with the Quick-RNA™ MiniPrep (Zymo Research, Freiburg, Germany). Total cellular RNA from tumor biopsies was extracted by peqGold TriFast™ (Peqlab, Erlangen, Germany). RNA integrity and quantity were assessed on an Agilent 2100 Bioanalyzer with a RNA 6000 Nano LabChip kit (Agilent Technologies, Waldbronn, Germany). Reverse transcription of 500 ng total cellular RNA with random hexamer primers was performed with an Omniscript RT kit (Qiagen). Expression of acidic ribosomal protein (ARP), PSA, IGF1, IGF1-R and IGFBP-3 was assayed on an iCycler iQ real-time detection system (Bio-Rad, Munich, Germany) with SsoFast EvaGreen supermix, (Bio-Rad). The 20 µl reaction from the kit was supplemented with 2 µl cDNA, 0.6 µM gene-specific primers (IBA, Göttingen, Germany). The following primers were used: IGF1, forward, 5'-TGGATGC TCTTCAGTTCGTG-3' and reverse, 5'-AGGGGTGC GCAATACATCT-3'; IGF1-R, forward, 5'-CCGAAGGTCTG TGAGGAAGA-3' and reverse, 5'-AATGGCGGATCTT CACGTAG-3'; IGFBP-3, forward, 5'-GAACTTCTCCTCCGA GTCCAA-3' and reverse, 5'-CTGGGACTCAGCACATTG AG-3'; PSA, forward, 5'-TGAACCAGAGGAGTTCTTGAC-3' and reverse, 5'-CCCCAGAATCACCCGAGCAG-3'; ARP, forward, 5'-CGACCTGGAAGTCCAACTAC-3' and reverse, 5'-ATCTGCTGCATCTGCTTG-3'.

Data analysis was performed according to the ΔΔCt-method (22). In cell culture and in animal experiments, ARP served as an internal control.

PSA secretion in serum from mice was measured with PSA Enzyme Immunoassay Test kit (GenWay Biotech, Inc., San Diego, CA, USA).

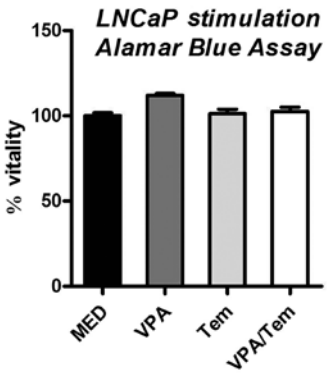

Figure 1. Effect of valproate and/or temsirolimus treatment on the vitality of pretreated LNCaP cells (n=6). Medium control was set as 100%.

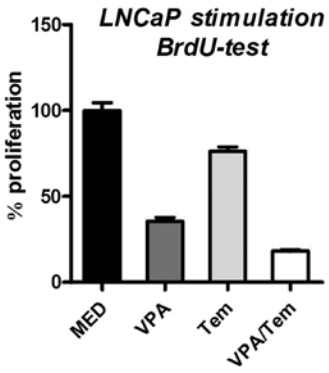

Figure 2. Effect of valproate and/or temsirolimus treatment on the proliferation of pretreated LNCaP cells (n=6). Medium control was set as 100%.

Histology. Sections of tumors were fixed with 4% formaldehyde in PBS for 72 h. The samples were processed according to standard procedures with a Leica TP1020 automatic tissue processor (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Paraffin-embedded tumor samples were dissected and stained with hematoxylin and eosin (HE) and immunostained against Ki-67 (23,24). For the calculation of the proliferation index, Ki-67 positive cells in correlation with the total cell number were counted in 10 fields of vision at x400 magnification.

Results

Valproate and temsirolimus reduce proliferation of LNCaP cells and act synergistically. In order to test whether the HDAC-inhibitor valproate or the mTOR-inhibitor temsirolimus or the combination of both drugs influence cell proliferation or vitality, we performed an Alamar Blue Assay and BrdU test with treated cells. In the Alamar Blue Assay, no significant difference to the untreated control was observed, indicating that cell vitality is not immediately affected by treatment with valproate or temsirolimus (Fig. 1).

By contrast, incubation of LNCaP cells with valproate resulted in a decreased cell proliferation by 64.6% (Fig. 2). Similarly, the stimulation with temsirolimus diminished cell proliferation by 23.9%. Furthermore, the combined treatment of LNCaP cells with both drugs resulted in a reduction of proliferation by 81.8% compared with untreated cells.

Expression changes in LNCaP cells following treatment with valproate, temsirolimus or a combination of both. To eluci-

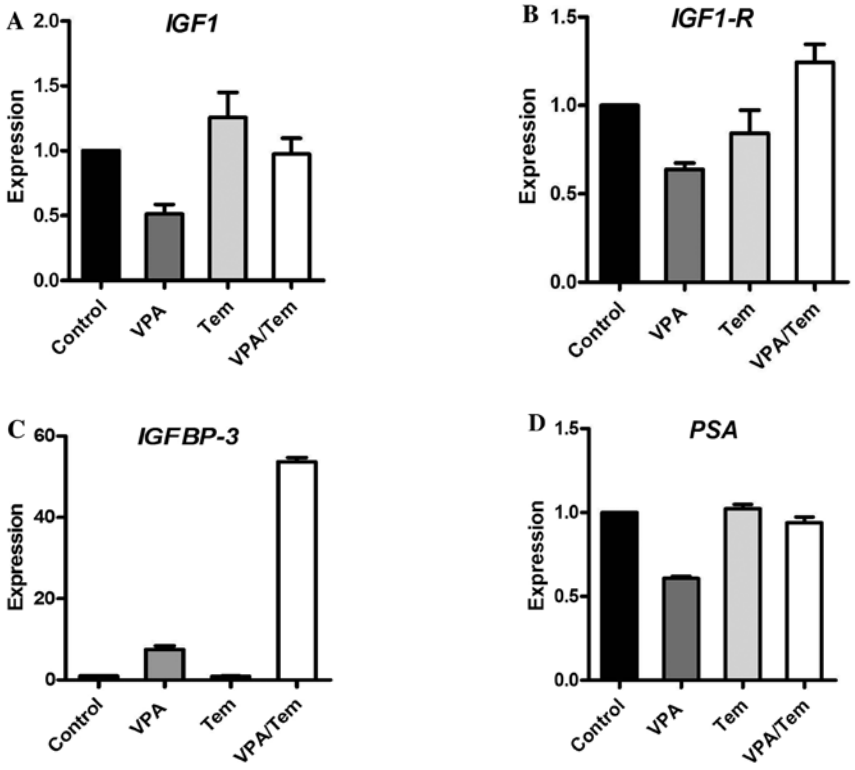

Figure 3. Expression of (A) IGF1, (B) IGF1-R, (C) IGFBP-3 and (D) PSA mRNA in LNCaP cells after treatment with valproate, temsirolimus or a combination of both (n=3). Medium control was set as 100%.

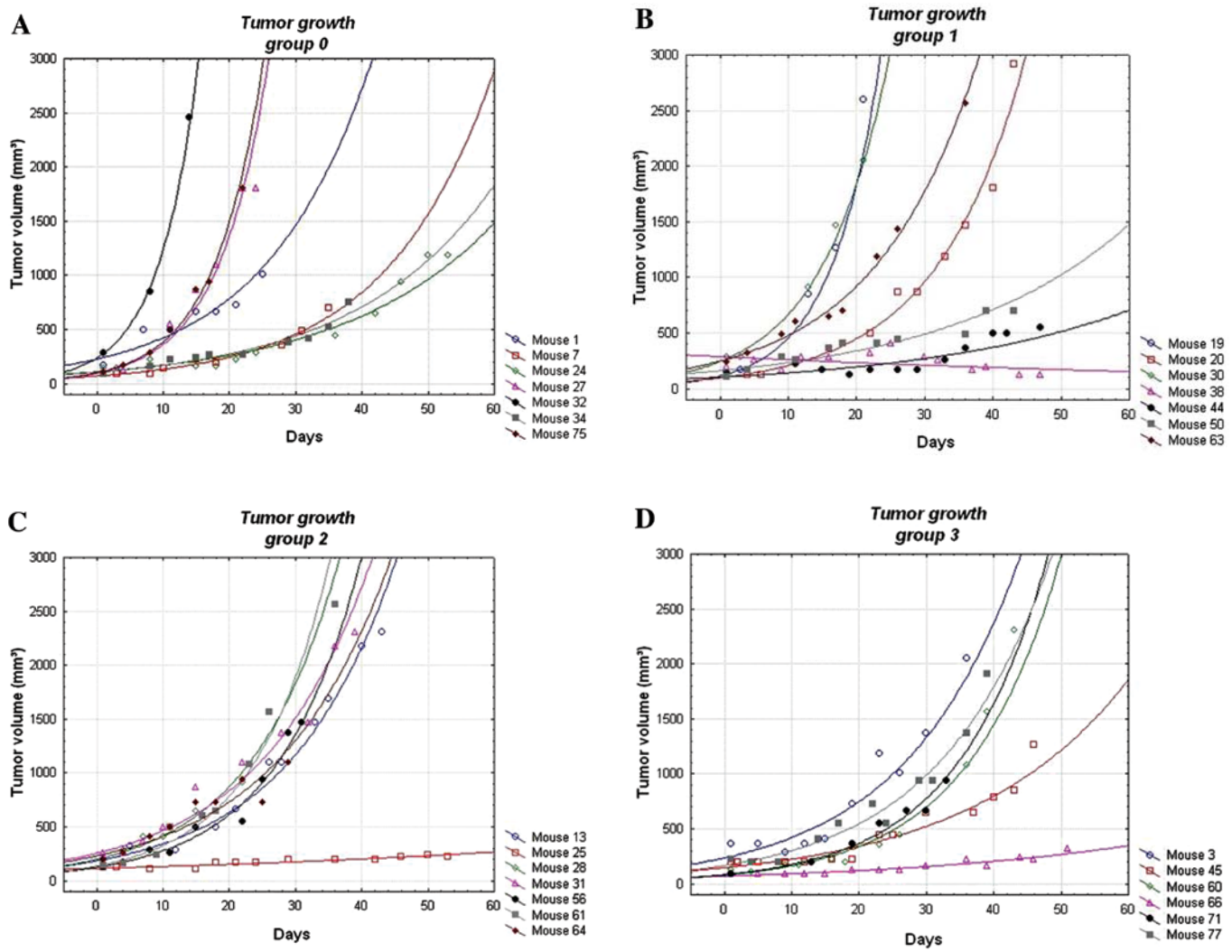

Figure 4. Tumor growth of implanted LNCap-derived tumors in nude mice. (A) Group 0, control; (B) group 1, valproate; (C) group 2, temsirolimus; (D) group 3, valproate and temsirolimus.

date if the reduced cell proliferation of treated LNCaP cells was associated with altered expression of key regulators of cell proliferation, we analyzed the mRNA expression of PSA, IGF1, IGF1-R and IGFBP-3 using real-time RT-PCR.

Incubation with valproate led to decreased expression of PSA, IGF1 and IGF1-R (Fig. 2). By contrast, treatment with temsirolimus or with both drugs combined did not influence the expression of these genes.

Consistent with a decreased proliferation, IGFBP-3 expression was elevated 7.5-fold upon valproate treatment. This effect was markedly enhanced by the combination of valproate and temsirolimus, resulting in a 53-fold increase in IGFBP-3 expression (Fig. 3).

Influence of valproate and temsirolimus on tumor growth in the mouse model. Next, we investigated whether the observed effect on cultured prostate carcinoma cells is also observed *in vivo* using LNCaP-derived tumors in a nude mouse model.

Therefore, 10^6 LNCaP cells were implanted subcutaneously into immune-suppressed nude mice. Of the 80 mice, only 31 (38.8%) developed tumors.

After reaching a distinct tumor volume (120 mm^3), mice were treated with valproate (800 mg/kg/day per os), temsirolimus (0,3 mg/kg weekly intravenously) or with a combination of both. A fourth group was left untreated and served as control.

Tumor growth (calculated by weekly measurements of tumor volume) did not show any significant differences between the distinct groups (Fig. 4). Variation in tumor growth within each group was rather high. Only the temsirolimus treated group exhibited, with one exception, a homogenous growth. Similarly, serum levels of human PSA, which was produced by the LNCaP cells of the tumors, did not differ between the groups (data not shown). The amount of secreted PSA serves as an indicator for the transcriptional activity of the androgen receptor.

Combined therapy of valproate and temsirolimus leads to a decreased proliferation capacity in vivo. Although we detected no difference in tumor growth (measured by the increase in tumor volume), histology preparations showed that the combined application of valproate and temsirolimus resulted in a significantly decreased proliferation capacity

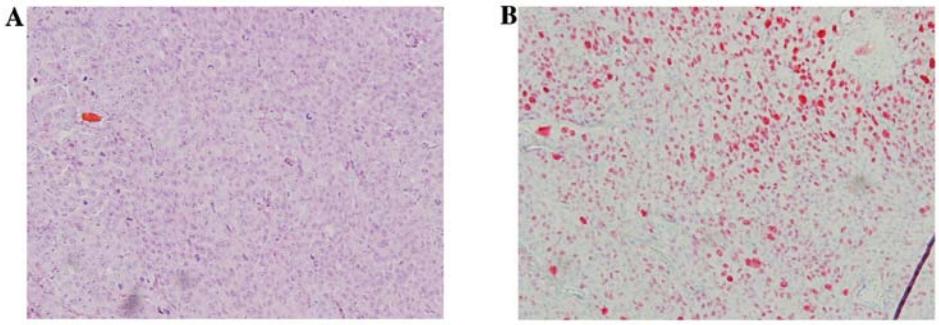

Figure 5. Tumor tissue group 0 (control). (A) HE and (B) Ki-67 staining. Magnification, x100; scale bar 100 μm.

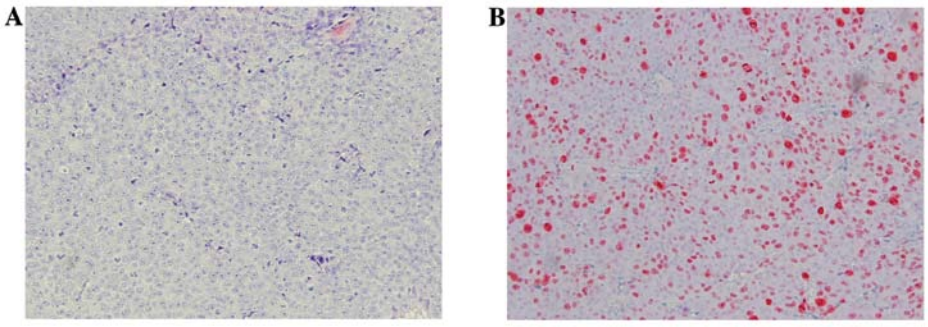

Figure 6. Tumor tissue group 1 (valproate). (A) HE and (B) Ki-67 staining. Magnification, x100; scale bar 100 μm.

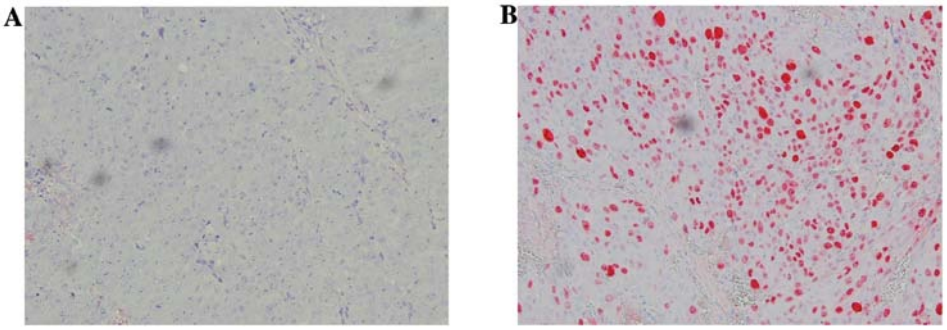

Figure 7. Tumor tissue group 2 (temsirolimus). (A) HE and (B) Ki-67 staining. Magnification, x100; scale bar 100 μm.

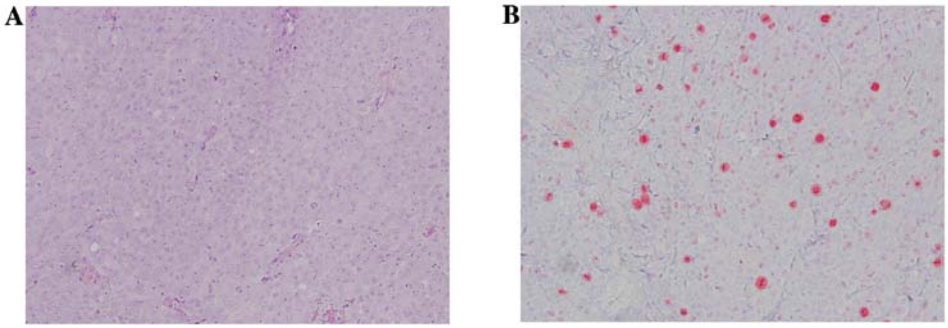

Figure 8. Tumor tissue group 3 (valproate and temsirolimus). (A) HE and (B) Ki-67 staining. Magnification, x100; scale bar 100 μm.

(49%) compared to untreated control animals (80%) or mice treated with valproate (81%) or temsirolimus (77%) alone (Figs. 5-8).

Expression changes in tumor cells following treatment with valproate, temsirolimus or a combination of both. Although no difference in tumor growth was observed, we investigated the

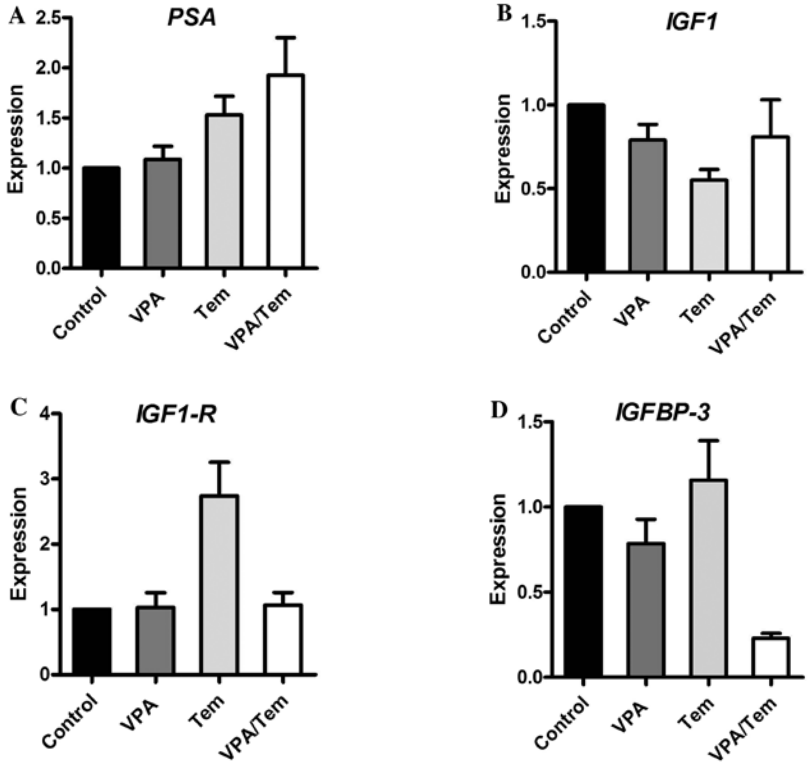

Figure 9. Expression of (A) PSA, (B) IGF1, (C) IGF1-R and (D) IGFBP-3 mRNA in tumor tissue after treatment with valproate, temsirolimus or a combination of both. Medium control was set as 100%.

mRNA expression of crucial regulators of cell proliferation in prostate carcinoma. Therefore, RNA was extracted from tumor samples and analyzed similarly to the cell culture experiments for the mRNA expression levels of PSA, IGF1, IGF1-R and IGFBP-3 via real-time PCR (Fig. 9). Valproate and the combination with temsirolimus enhanced the expression of PSA. IGF1 was reduced upon treatment with valproate and temsirolimus. The expression of IGF1-R was only increased by temsirolimus. In contrast to our cell culture experiments, the combination of valproate and temsirolimus reduced the expression of IGFBP-3.

Discussion

In the present study, we showed that valproate and temsirolimus mediate a decrease in the proliferation potential of a cultured prostate carcinoma cell line. Furthermore, the enhanced effect of a combined treatment suggests that both drugs act synergistically. Valproate has been described to inhibit tumorigenesis by reduction of tumor growth and induction of cell differentiation in different cell lines, such as neuroblastoma cells (SH-SY5Y) and a breast cancer cell line (MT-450) (9,25,26). In a previous study, we showed a fundamental mechanism underlying this phenomenon: valproate inhibits the activity of HDACs in LNCaP cells, leading to an increased expression of ERβ (10,11). ERβ itself exhibits antiproliferative, anti-invasive and pro-apoptopic capacities and is downregulated in prostate carcinoma (27-31). In addition, valproate is a negative regulator of the cell cycle: treatment with valproate increases the amount of G0/G1 phase prostate carcinoma cells and reduces the expression of Cdk1 (32). Decreased Cdk1 levels in turn result in a reduction of androgen receptor transcription (33). On the other hand, an antiproliferative influence has been reported for various mTOR inhibitors *in vitro* and *in vivo* as well (34). For instance, the application of rapamycin significantly reduces the growth rate of induced prostate carcinomas in a mouse model (35). Furthermore, Fung *et al* (36) showed a decreased proliferation of LNCaP cells upon treatment with temsirolimus, connected with a reduction in S-phase cells and an increase in G1-phase cells. Similar results have also been achieved with another mTOR inhibitor, RAD001 (32). The reduction of proliferation of LNCaP cells treated with a combination of valproate and temsirolimus observed in this study is in line with data from Wedel *et al* (32), who reported a synergistic effect of valproate with RAD001 on the inhibition of the cell cycle.

We further showed that the application of valproate on LNCaP cells results in a decrease of PSA expression, which is most likely due to an increase in ERβ expression (12). PSA is frequently used to asses the activity of prostate carcinoma and therefore a valid marker for tumorigenesis and diagnosis (37). A key regulator in tumorigenesis is the IGF-signaling pathway, which influences crucial processes such as cell proliferation, differentiation, transformation and apoptosis (38). Two regulatory mechanisms are essential for triggering the IGF activity: IGFBP-3 binds IGF1 and thereby competes with the IGF-receptor whereas PSA cleaves and inactivates IGFBP-3, thus increasing the pool of functional IGF (39). IGF1/IGF1-R-signaling enhances the metastatic capacity of prostate carcinoma cells (40,41) and facilitates the nuclear import of the androgen-receptor in the absence of androgens (42,43). In a previous study, IGF1 and IGFBP-3 were found to be correlated with advanced prostate cancer (44). Thus, IGF1 is a key mediator for the development of a castration-resistant prostate carcinoma, as it is not directly targeted by the currently used anti-androgen therapy. We found that treatment with valproate

leads to a decreased expression of IGF1 and IGF1-R, whereas the expression of IGFBP-3 is enhanced. Furthermore, the combined application of valproate and temsirolimus further potentiates this effect. By contrast, temsirolimus alone does not affect IGFBP-3 expression. In addition to its inhibitory effects on IGF-signaling, IGFBP-3 also exhibits IGF-independent antiproliferative and pro-apoptotic abilities (19,20).

We showed that the simultaneous treatment of a prostate carcinoma cell line with the HDAC-inhibitor valproate and the mTOR inhibitor temsirolimus leads to synergistic antiproliferative and pro-apoptotic effects by modulating the IGF1-signaling pathway. However, why the application of temsirolimus alone has no effect and the underlying mechanisms for the synergistic effects of the combined therapy, remain unclear.

To investigate whether the observed effect of valproate and temsirolimus on cultured prostate carcinoma cells is sufficient to inhibit tumor growth *in vivo*, we implanted LNCaP cells into nude mice. However, we observed no significant difference in tumor volume over the time of drug administration between the treated and untreated groups. Which concentration of the drug actually reaches the transformed cells due to its specific bioavailability has yet to be clarified. For valproate it has been shown that the dose rate we used in this study is sufficient to obtain a blood concentration, which is used in patients (45); however, different vascularization of the tumor may also influence the concentration of the drug at its destination. To our knowledge, for temsirolimus, no data are available about its bioavailability and its final concentration in targeted tissues.

In contrast to our finding, valproate has been shown in other studies to inhibit tumor growth in human stomach and liver carcinomas (46,47).

Contradictory reports have been published regarding the *in vivo* effect of mTOR inhibitors. The mTOR inhibitor RAD001 was able to reduce the growth of prostate carcinoma in mice as well as prostate carcinoma in mice tibiae (35,48). By contrast, the treatment of LNCaP-derived tumors in SCID mice with RAD001 did not affect tumor growth, which is in line with our results (49).

Consistent with unchanged tumor volumes, levels of human PSA in the blood of treated versus untreated mice also showed no differences. Therefore, we concluded that the activity of the androgen receptor is not permanently altered upon drug treatment in our experiment. This is in contrast to our findings from the cell culture experiment, where valproate reduced the expression of PSA 2.9-fold, whereas temsirolimus and the combined application of both drugs did not show any effects.

Whether the potential of valproate on PSA expression is not sufficient to reduce PSA levels in the blood or whether other mechanisms, which are not present *in vitro,* interfere, remain to be clarified.

In addition, the results from the expression analysis of PSA, IGF1, IGF1-receptor and IGFBP-3 differ from our cell culture experiments.

While PSA mRNA is decreased in cultured prostate carcinoma cells, its expression *in vivo* is increased by 53% upon treatment with temsirolimus and combined therapy with valproate and temsirolimus even potentiates this phenomenon. However, this elevated expression is not reflected by an increase in serum-PSA. Clearly, expression alterations induced by short-term stimulation of cultured cells do not persist in long-term *in vivo* treatments. In addition, the mRNA expression of IGF-axis genes does not reflect tumor growth features *in vivo*.

Although we did not observe any significant growth inhibition of prostate carcinoma cell-derived tumors in our nude mouse model upon treatment with valproate, temsirolimus or a combination of both, staining against the cell cycle marker Ki-67 in histological sections of the tumors showed a remarkable reduction in the proliferation potential when the mice were treated with a combination of valproate and temsirolimus. By contrast, tumors from untreated animals or mice treated with valproate or temsirolimus alone showed a significant higher proliferation potential. This is in line with our finding from the cell culture experiments, where the combined therapy exhibits synergistic effects. A similar phenomenon was observed for the combination of valproate with another mTOR inhibitor, RAD001, in cultured prostate carcinoma cells (32). However, we can only speculate why the reduced proliferation potential does not result in a reduced tumor growth. One possibility is that the period of treatment was too short, transformed cells proliferate in the beginning, without interference of a drug, and reach a steady state situation upon treatment with the valproate-temsirolimus combination. This is reflected by a reduced Ki-67-staining in tumor sections. Prolonged and extended studies are needed to clarify whether these tumors would finally arrest growth.

We hypothesize that the combined therapy of prostate carcinoma with the HDAC inhibitor valproate and the mTOR inhibitor temsirolimus exhibits synergistic effects on cell proliferation and apoptosis and is thus potentially capable of inhibiting tumor growth. The mTOR kinase is known to be the main signal integrating point receiving inputs via the PTEN/PI3K pathway through the action of Akt. In castration-resistant prostate cancer, mutated PTEN is expressed opening the PTEN/PI3K/Akt pathway for constitutive activation of anti-apoptotic mechanisms and activation of the mTOR pathway in the absence of androgens. Clinical trials with mTOR inhibitors in advanced prostate cancer led to the conclusion that it is likely that such agents will need to be combined with other therapies (50). Here, we combined the mTOR inhibitor temsirolimus with the HDAC inhibitor valproate with anti-androgen capacities, as we previously demonstrated (12). However, our *in vivo* data show that further studies are warranted to evaluate the optimal conditions (dose rate, application form, formulation) for the application of these two drugs in the therapy of prostate carcinoma.

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